Supporting Information

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SI Materials and Methods

Evaluation of Copy Number Changes of *PRDM1*. To determine *PRDM1* copy number loss, we used quantitative RT-PCR (qRT-PCR) on genomic DNA, and specifically measured *PRDM1* in comparison with a housekeeping gene, *RPL13A*. The qPCR experiment was performed with the DyNAmo HS SYBR Green qPCR Kit (Finnzymes Inc.) to detect the fluorescence of the amplicons. The ΔΔ Ct method was used for the data analysis. The cutoff for monoallelic deletion was set at 0.75-fold of the value of the calibrator sample (i.e., tonsil genomic DNA) in terms of the ratio of *PRDM1* to *RPL13A*. Primers used to test *PRDM1* gene copy number include *PRDM1* forward: *TGCTTCAGTTCTCTCTAGCCCTCT; PRDM1* reverse: *ACGCTGTACTCTCTCTTTGGGACA; RPL13A* forward: *ACCACCACCACCTGCACTTATTCT; RPL13A* reverse: *TGGGTCTTGAGGACCTCTGTGTAT.*

Quantitation of *PRDM1* mRNA. *PRDM1* mRNA expression in NKCL cases was measured using qRT-PCR. The expression of *PRDM1*α and total *PRDM1* (α + β) in resting and IL-2–stimulated primary NK cells at 2-d intervals until day 6 was determined with qRT-PCR as described previously (1). The expression of *PRDM1*α and total *PRDM1* (α + β) was detected in primary human NK cells derived from the coculture of peripheral blood lymphocytes and K562-Cl9-mb21, a K562 line modified by the retroviral transduction of 4-1BBL, CD86, and IL-21, a kind gift from Dean A. Lee (MD Anderson Cancer Center, Houston).The primer pairs were designed across exon–exon junctions, and melting curves were used to assess the specificity of amplifications. For PRDM1-FL, the forward primer was located in PRDM1 CDS and the reverse primer was located in the flag (FL) tag. The primers for *PRDM1* and other genes tested with qRT-PCR are as follows: *PRDM1-FL* forward: TCATGAAGTTGCCTCCCAGCAA; *PRDM1-FL* reverse: TCATCGTCGTCCTTGTAATCAGCG; *PRDM1* forward: TTTCGGCCAGCTCTCCAATCTGAA; *PRDM1* reverse: CAGACCTGGCATTCATGTGGCTTT; *PRDM1α* forward: ACAGTTCCTAAGAACGCCAACAGG; *PRDM1α* reverse: AAGCCGTCAATGAAGTGGTGAAGC; *RPL13A* forward: ACCGTCTCAAGGTGTTTGACG; *RPL13A* reverse: GTACTTCCAGCCAACCTCGTG; *CCNG1* forward: ACTGCACGGCAATTGAAGCATAGC; *CCNG1* reverse: AGGTTGTGGAGAAAGGCTTCAGAG; *CCNG2* forward: AGTGTTCCTGAGCTGCCAACGATA; *CCNG2* reverse: AAGGTGCACTCTTGATCACTGGGA; *MYC* forward: TGCAGCTGCTTAGACGCTGGATTT; *MYC* reverse: GTCGAGGTCATAGTTCCTGTTGGT; *41BBL* forward: TCCCGAGCTTTCGCCCGACGAT; *41BBL* reverse: GGGCCCATCGATCAGCAGAACATTT; *TNFα* forward: AGTGACAAGCCTGTAGCCCATGTT; *TNFα* reverse: GTTATCTCTCAGCTCCACGCCATT; *TNFβ* forward: TCAAACCTGCTGCTCACCTCATTG; *TNFβ* reverse: AGAAACCATCCTGGAGGAAGGCAC.

Methylation Analysis of the *PRDM1* Promoter Region. We used CpG Island Searcher, a program that uses an improved version of Gardiner-Garden sequence criteria (2) for CpG island prediction with the following parameters: (*i*) GC content >55%, (*ii*) ratio of observed-to-expected number of CpG dinucleotides >0.65, and (*iii*) length >500 bp (3).CpG Island Searcher predicted a CpG island of 638 bp, spanning a genomic region around the *PRDM1*α transcription start site (TSS; −311 and +327 of TSS) when a 28,620-bp genomic region that includes 5,000 bp upstream of the *PRDM1*α TSS and all exons and intronic sequences was screened. The methylation pattern of the *PRDM1*α promoter (−786 and +1 of TSS) was determined by bisulfite treatment of 1 μg of genomic DNA with the EZ DNA Methylation-Direct Kit (Zymo Research) (1). A total of 20 ng of bisulfite-modified DNA was used as template for PCR reactions using FastStart Taq DNA Polymerase (Roche Diagnostics) as per the manufacturer's instructions. The methylation percentage (average of three technical replicates) of each CpG was determined by the pyrosequencing methodology using the Qiagen Pyromark Q24 pyrosequencer according to the manufacturer's recommendations. Primers used in the methylation analysis are as follows: region 1 forward: TTAATAGAGGATTGATTGGGTTG, region 1 reverse: CCCAAAACCCACAAACTAAC; region 1 pyrosequencing: GGGTTGGAGGAAAAGTTGGT; region 2 forward: AGTTAGTTTTGTTAAGTTTGA; region 2 reverse: CTAATCCTCAAACTATTATATATATAA; region 2 pyrosequencing: AGTTAGTTTTGTTAAGTTTGAA; region 3 forward: ATTTGAGTTGAGAAATTAGAAATT; region 3 reverse: ACTTCCCTTTAAAAACACTTAA; region 3 pyrosequencing: AAGTTTTTTTAATTTAAGGAA; region 4 forward: GTGTTTTTAAAGGGAAGTAAGAAG; region 4 reverse: CCCTCCCTACTTAAAATTTCCTTAA; region 4 pyrosequencing: GTTAATGTTGAAATATATATG.

Mutation Analysis of *PRDM1*. *PRDM1* has seven exons, of which six are coding. We searched for mutations in the *PRDM1* gene in all tumor cases using previously tested primer sets (4). The amplicons for sequencing include the genomic coding sequences as well as the intron–exon junctions of *PRDM1*. Briefly, each amplicon was generated from 30 ng of genomic DNA with PCR, purified with Microcon YM-50 columns (Millipore), and used for Sanger sequencing. Sequencing results were analyzed with Vector NTI (Invitrogen) by aligning the sequences obtained from cases with that of PRDM1 Ref Seq in the National Center for Biotechnology Information (NCBI) database. Sequencing was repeated in the reverse direction in cases having ambiguous results with forward sequencing. The nucleotide alterations found were also screened through the NCBI SNP database to determine if the alteration is a known SNP.

Reconstitution of PRDM1 and hTERT in NK Cell Lines. A FLAG-tagged *PRDM1* retroviral construct was obtained from pCDNA3.1−(NCBI NM_001198.2) and subcloned upstream of the internal ribosome entry site (IRES) sequence of murine stem cell virus-IRES-GFP (pMIG). Similarly, hTERT was PCR cloned into the pMIG plasmid using pBABE-puro-hTERT as the template. Retroviruses were generated by cotransfection of 3 μg of empty plasmid vector or vectors containing *PRDM1*α, or hTERT constructs with 3 μg of the packaging plasmid pCL-Ampho using Turbofect (Fermentas) into 60–70% confluent 293T cells seeded 24 h earlier for generation of the retrovirus. At 24 h after transfection, the medium was replaced with 3 mL fresh medium, and 48 h after transfection, the supernatants were collected, spun down at 2,095 × *g* for 5 min to remove the cellular debris, and filtered with a 0.45-μm filter. A total of 500,000 cells were mixed with 1 mL retroviral supernatant in 12-well plates, with 10 μg/mL Polybrene (Chemicon-Millipore) added. Cells were then centrifuged at 524 × *g* for 90 min at 4 °C. After centrifugation, cells were incubated in a humidified, 5% CO_2 incubator at 37 °C for 10 h. At 60 h after transduction, the retroviral supernatant from 293T cells was collected, and the transduction was repeated. At 10 h after the second transduction, the transduced cells were spun down and resuspended in the regular NK cell growth medium.

Apoptosis Assay. Apoptosis of retrovirally transduced cells was quantified using a FACSCalibur flow cytometer (BD Biosciences) after staining the cells with Annexin V-PE (Apoptosis Detection Kit; BD Pharmingen) according to the manufacturer's instructions. The ratio of apoptotic cells was determined by calculating the ratio of Annexin V staining in GFP(+) cells. The percentage of Annexin V+ cells was calculated in GFP(+) and GFP(−) populations for vector- or PRDM1-transduced KHYG1 cells after 2 d of treatment with limiting doses of IL-2 and normalized to the percentage of Annexin V staining in 100 IU IL-2–treated KHYG1 cells to test the role of IL-2 concentration on apoptosis of NK cells.

Cell Cycle Assay. The cell cycle profile of retrovirally transduced cells was analyzed using Hoechst 33342 staining (H3570; Invitrogen). The cells were washed once with PBS and resuspended in buffer containing 1× PBS + 0.1% BSA at 0.5×10^6 cells/mL. Hoechst dye was added to the cells at a final concentration of 10 μg/mL. The cells were incubated at 37 °C for 15 min and then analyzed with a BD LSR II flow cytometer.

Evaluation of the Role of PRDM1α in Malignant NK Cell Lines and Primary NK Cells by Tracking the Percentage of GFP(+) Cells in Transduced NK Cell Lines or Primary NK Cells. The percentage of GFP(+) cells in the forward and side scatter gated-transduced population was quantified in retrovirally transduced malignant NK cell lines and primary NK cells using FACSCalibur cytometer to show the negative or positive selection of the PRDM1-transduced PRDM1-null NK cell lines or primary NK cells transduced with PRDM1 siRNA, respectively. Untransduced NK cells were used as the negative control. For the IL-2 treatment assay, the percent decrease in GFP was calculated by dividing the percentage of GFP(+) cells of the PRDM1-transduced NK cells with the vector-transduced NK cells for the corresponding IL-2 concentration for the same time point. The percent GFP determined before the initiation of the IL-2 assay was used for normalization.

Design and Expression of siRNA for PRDM1 Knockdown in Primary Human NK Cells. Primary human NK cells were obtained by coculturing peripheral blood lymphocytes (PBLs) with 100 Gr irradiated K562-Cl9-mb21 (first stimulation) at a 1:2 ratio. After 7 d, cocultured cells were mixed at a 1:1 ratio with irradiated K562-Cl9-mb21 cells. By day 14, the purity of NK cells was 96%, as determined by CD56 and CD3 staining by flow cytometry. At day 13, NK cells were transduced with pMIG or PRDM1 siRNA-mir30-pMIG (5). NK cells were transduced in two successive days. At 24 h after the second transduction, cells were resuspended in NK cell expansion medium. The result was represented as percent change by normalizing the later time points with the earliest time point for each sample.

Transfection of HEK293T Cells and the Luciferase Reporter Assay for Testing the PRDM1 shRNA. Sixty-thousand HEK 293T cells were seeded on 24-well plates and transfected the next day with ExGen 500 transfection reagent (Fermentas) per the manufacturer's instructions. A total of 500 ng U6-PRDM1-shRNA-KSII+, pGL3-P-PRDM1 (50, 100, or 200 ng) and pRL-SV40 (1/25th of the amount of pGL3-P-PRDM1) was cotransfected into HEK 293T cells. Empty U6-KSII+ vector was added to keep the total plasmid DNA at 1 μg for all transfections. Luciferase activity was determined 62 h later with the Dual-Luciferase Reporter Assay System (Promega, Inc.) per the manufacturer's instructions. ATG5 shRNA was used as the negative control. In the second experiment, 950 ng of PRDM1 shRNA-mir30 construct was cotransfected with 52 ng of pGL3-P-PRDM1+ pRL-SV40 (25:1), and relative luciferase activity was determined as described above.

Western Blot. Western blotting was performed as described previously (1) using anti-PRDM1 (NB600-235H; Novus Biologicals) and anti–α-tubulin (T6074; Sigma Aldrich) antibodies.

1. Iqbal J, et al. (2009) Genomic analyses reveal global functional alterations that promote tumor growth and novel tumor suppressor genes in natural killer-cell malignancies. *Leukemia* 23:1139–1151.
2. Gardiner-Garden M, Frommer M (1987) CpG islands in vertebrate genomes. *J Mol Biol* 196:261–282.
3. Takai D, Jones PA (2002) Comprehensive analysis of CpG islands in human chromosomes 21 and 22. *Proc Natl Acad Sci USA* 99:3740–3745.
4. Braggio E, et al. (2009) Identification of copy number abnormalities and inactivating mutations in two negative regulators of nuclear factor-kappaB signaling pathways in Waldenstrom's macroglobulinemia. *Cancer Res* 69:3579–3588.
5. Kruschinski A, et al. (2008) Engineering antigen-specific primary human NK cells against HER-2 positive carcinomas. *Proc Natl Acad Sci USA* 105:17481–17486.

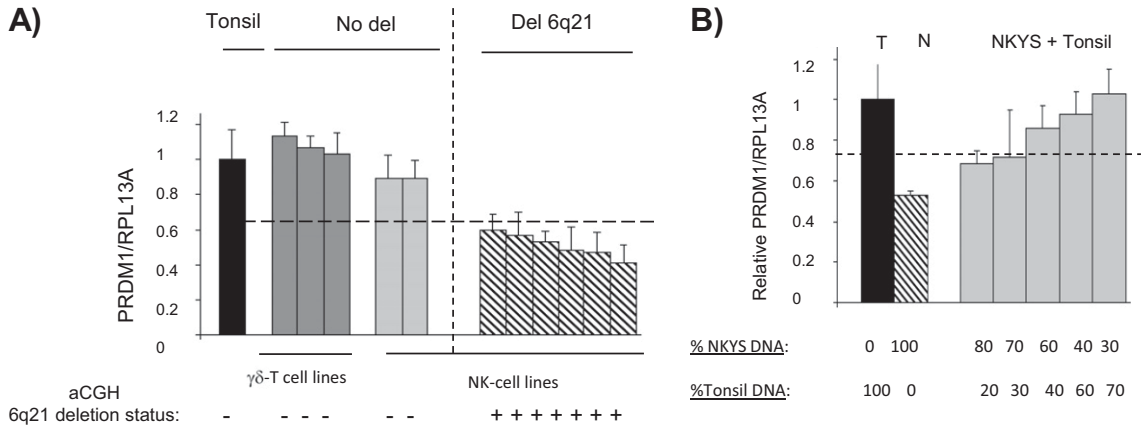

Fig. S1. Testing the utility of the qPCR method for the detection of copy number loss and estimation of the influence of stromal contamination on analysis of *PRDM1* copy number in NKCL cases. (*A*) Monoallelic deletion of *PRDM1* is frequently observed in malignant NK cell lines. Validation of the monoallelic deletion of *PRDM1* identified with BAC a-CGH using qPCR in NK cell lines. Three γδ T-cell lines and two NK cell lines with no del(6)(q21) were compared against six NK cell lines with del(6)(q21). Human tonsil genomic DNA was used to define the cutoff for monoallelic deletion. (*B*) Potential contributions of the stromal DNA in tumor samples to the qPCR results were tested by mixing the genomic DNA of an NK cell line with del(6)(q21) with that of tonsil DNA. For each sample, *PRDM1/RPL13A* was calculated from the average of at least three independent amplifications for both genes. The dashed horizontal line represents the cutoff value for the assignment of *PRDM1* deletion status of the NK cell lines. Standard bars represent the SD for at least three different replicates for PRDM1 and RPL13A. del 6q21, samples with 6q21 deletion; N, NKYS cell line; no del, samples without 6q21 deletion; SNT, γδ T-cell lines; T, tonsil genomic DNA.

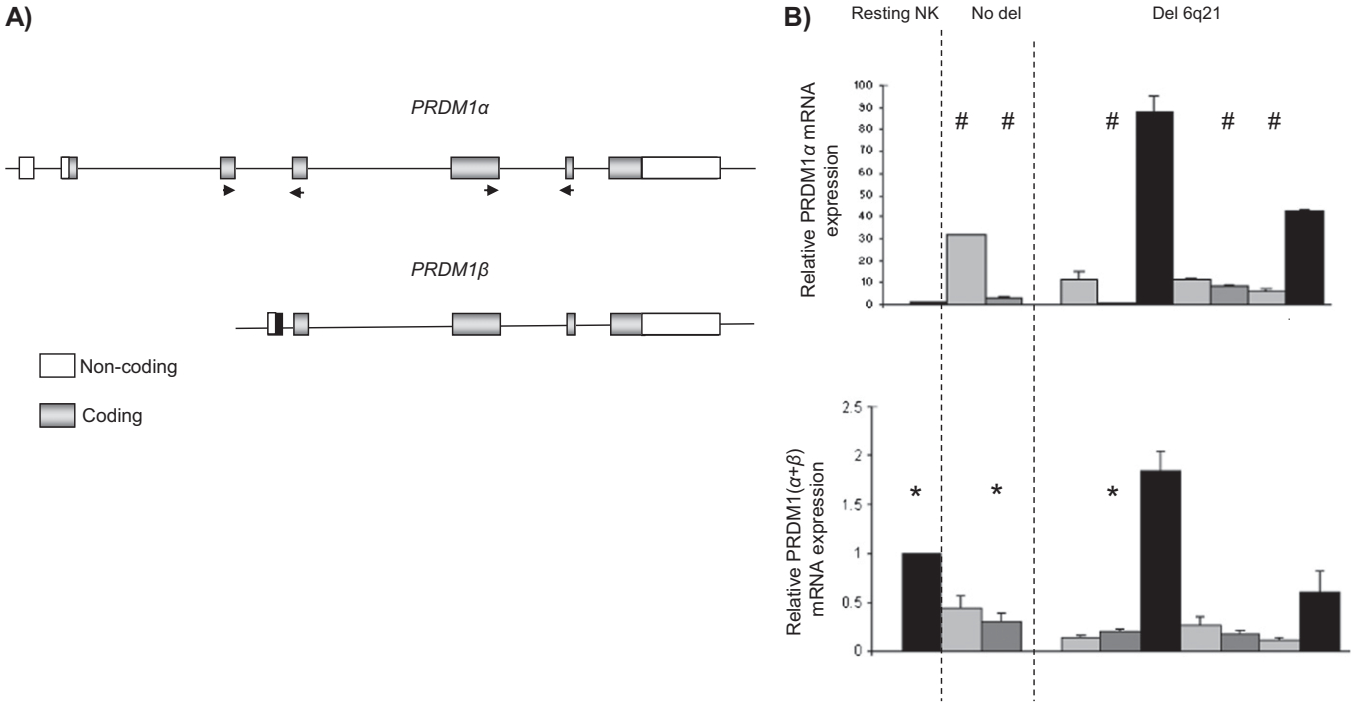

Fig. S2. (Continued)

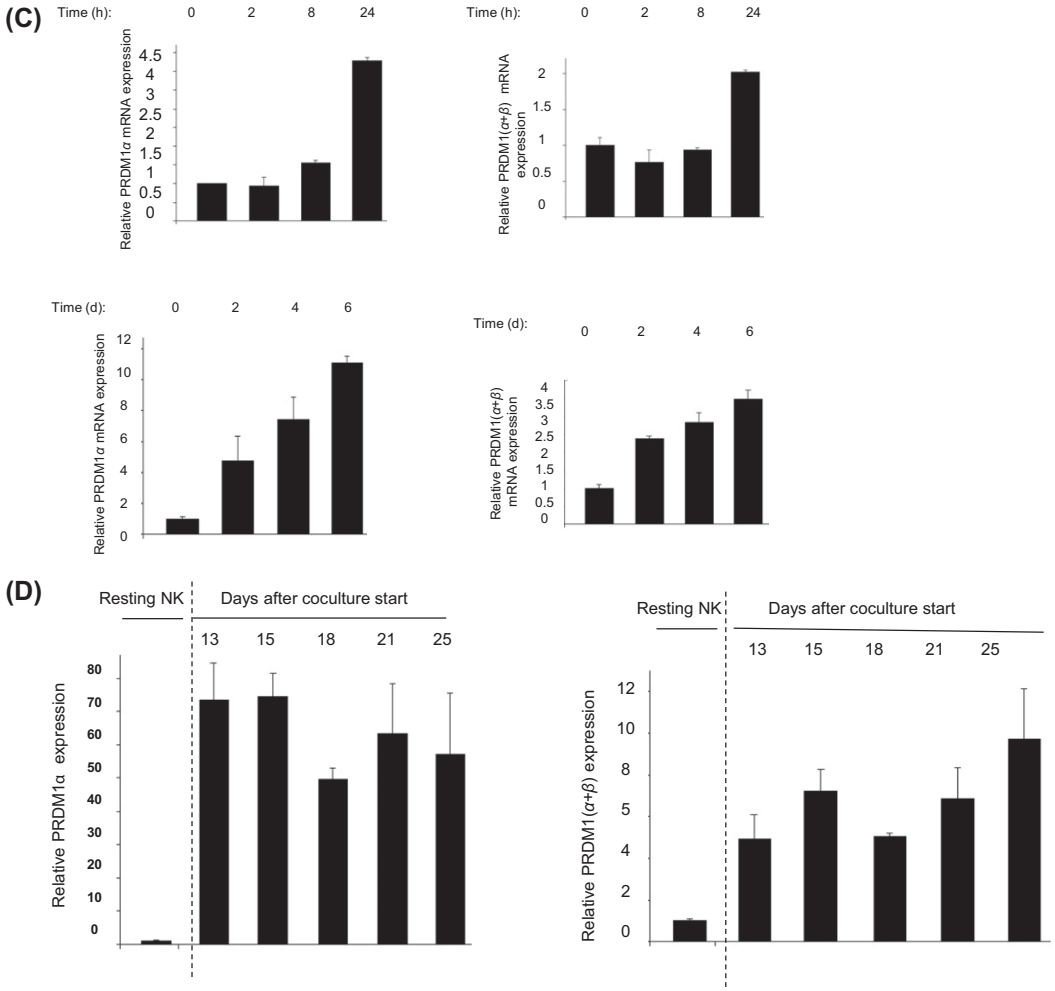

Fig. S2. Quantification of *PRDM1*α and *PRDM1* (α + β) mRNA in NK cell lines and primary human NK cells. (*A*) The *PRDM1*α and *PRDM1*β transcripts and the locations of the qRT-PCR primers used to measure *PRDM1*α and *PRDM1* (α + β) are shown. (*B*) *PRDM1*α and *PRDM1* (α + β) expression in NK cell lines determined with qRT-PCR. The samples with discordant *PRDM1*α or *PRDM* (α + β) levels are indicated with asterisks. NK cell lines with promoter methylation are shown with the pound sign. (*C*) *PRDM1* is up-regulated in normal NK cells in response to IL-2. Relative quantification of the *PRDM1*α and *PRDM1* (α + β) transcripts with qRT-PCR in peripheral blood NK cells cultured with 100 IU IL-2 for 24 h (*Upper*) and with 200 IU IL-2 in 2 d intervals up to 6 d (*Lower*). Each figure is representative of two independent experiments with two technical replicates for each data point (n = 2). (*D*) PBLs were stimulated with K562-Cl9-mb21 twice at day 0 and day 7 as described in *Materials and Methods*. qRT-PCR was applied on cocultured cells to quantify *PRDM1*α (*Left*) and *PRDM1* (α + β) (*Right*) mRNA starting on the 13th day of the initial stimulus. At day 14, most of the K562-Cl9-mb21 cells had been killed, and the cocultured cells were 95.8% CD56+CD3− NK cells. Each data point represents two technical replicates for *PRDM1* or *RPL13A*. Apart from the data for day 13, each plot is representative of two independent experiments.

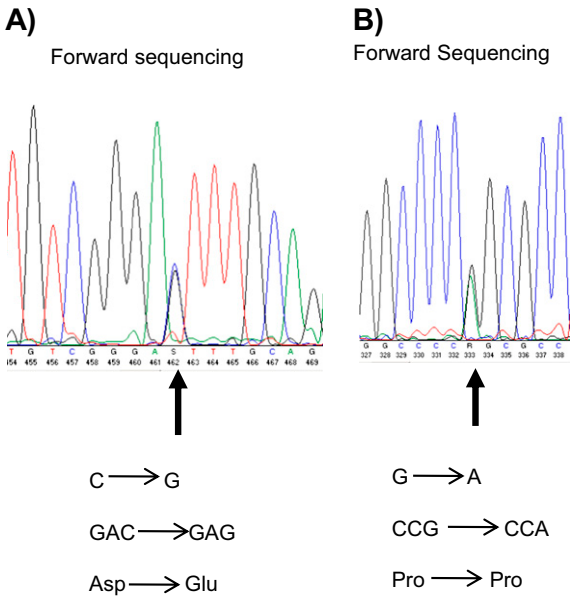

Fig. S3. SNPs identified in the genomic sequence of *PRDM1* in NKCL cases. (*A*) The missense SNP found in an NKCL case. (*B*) The silent SNP found in two other NKCL cases.

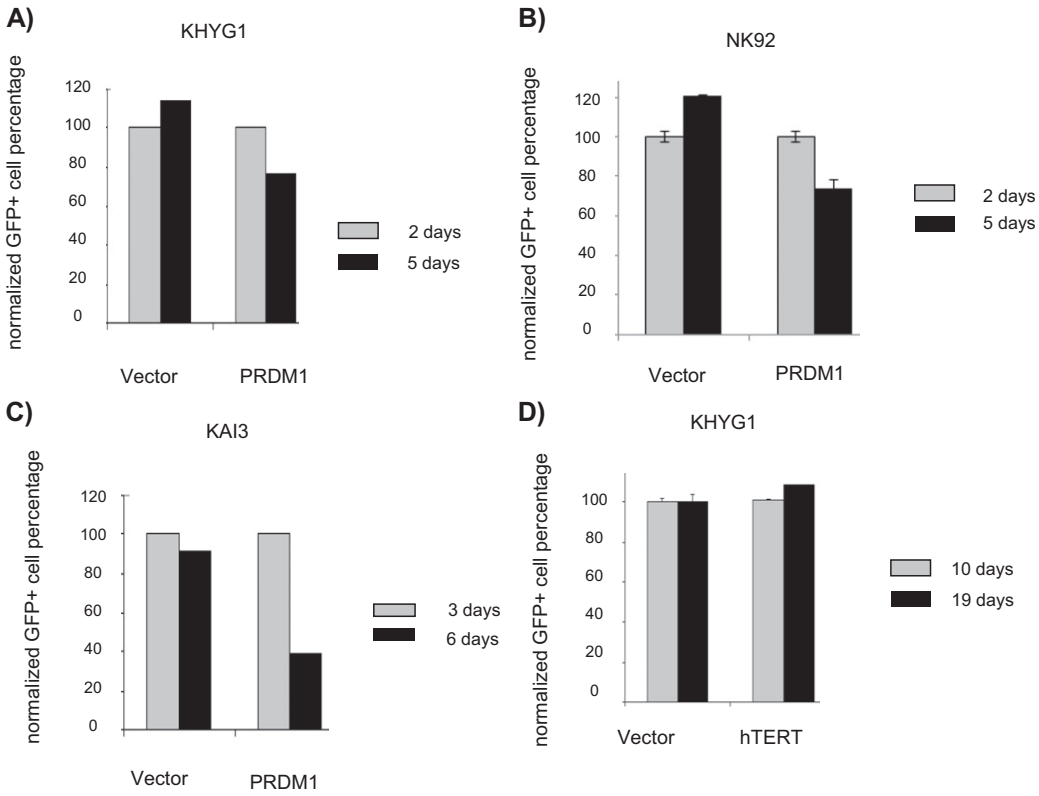

Fig. S4. Quantification of the percentage of GFP(+) cells at different time points after transduction with the vector or PRDM1. Comparison of the change in the percentage of GFP in vector- or PRDM1-transduced KHYG1 (*A*), NK92 ($n = 2$) (*B*), or KAI3 (*C*) cells normalized to the percent of GFP for the earlier time point. (*D*) hTERT-transduced KHYG1 cells were used as control. The error bars represent SD of two biological replicates.

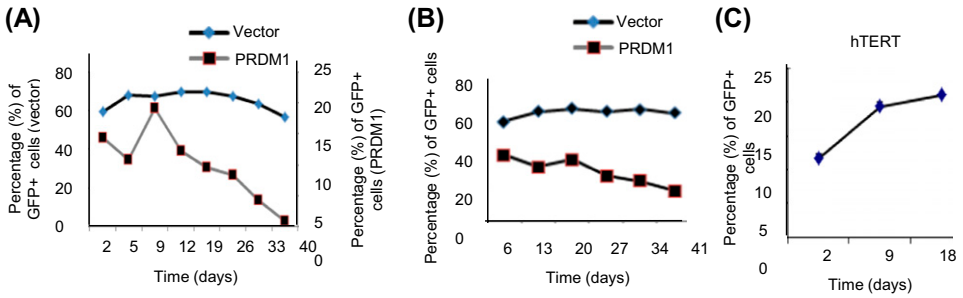

Fig. S5. Ectopic expression of PRDM1 exerts negative selection pressure in NK cells on long-term culture. The percentage of GFP(+) cells was quantified in vector-, PRDM1-, or hTERT-transduced KHYG1 (*A* and *C*) and NKYS (*B*) cells with flow cytometry on long-term culture. The time points represent the days after retroviral transduction.

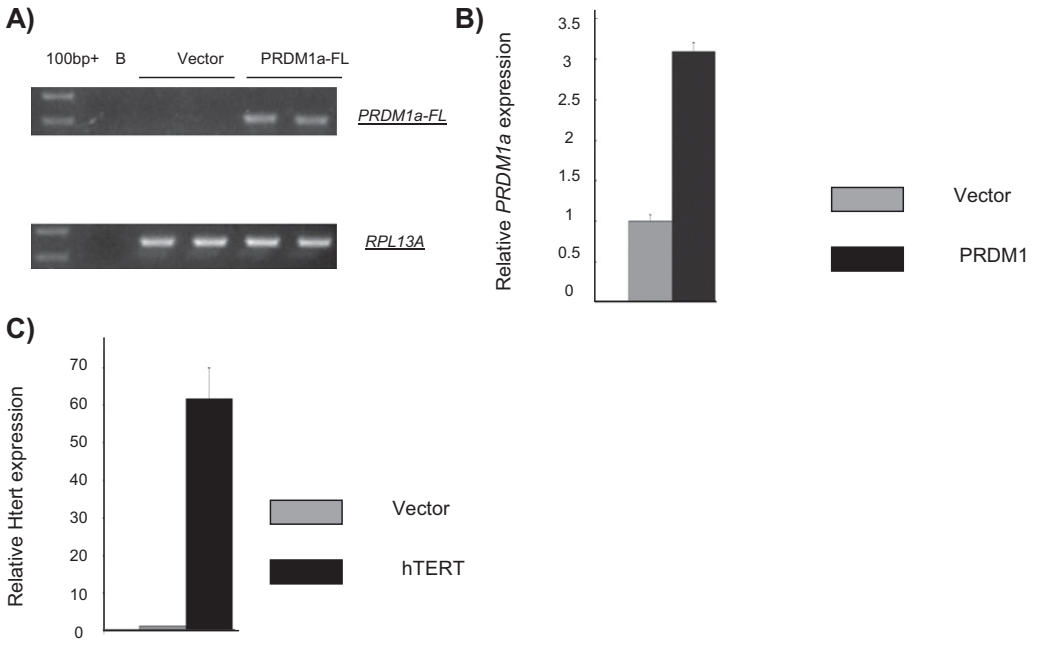

Fig. S6. Ectopic expression of PRDM1 and hTERT in KHYG1 cells. PRDM1 or hTERT expression was tested on transduced KHYG1 cells with qRT-PCR using (*A*) PRDM1α-FL– or (*B*) *PRDM1*α-specific primer sets on PRDM1-transduced KHYG1 cells. (*C*) hTERT expression was assayed on KHYG1 cells transduced with hTERT using qRT-PCR.

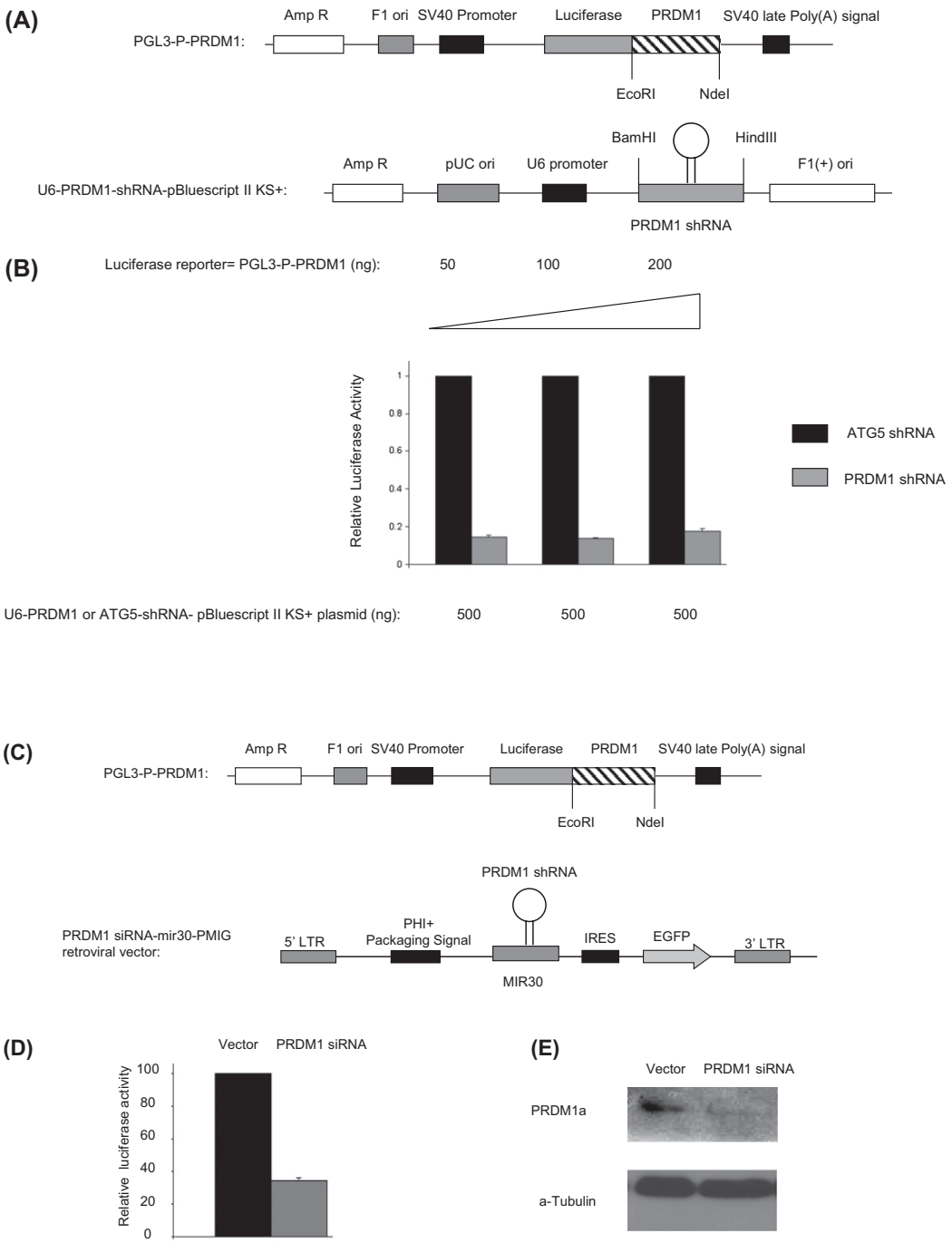

Fig. S7. Demonstration of the functionality of the PRDM1 shRNA. (*A*) A previously reported PRDM1 siRNA nucleotide sequence (1) was chosen to knock down *PRDM1* in human primary NK cells. (*B*) PRDM1 shRNA F and R (IDT, Inc.) sequences were designed based on the manufacturer's instructions (ThermoScientific) and cloned into a modified pBluescript KSII+ vector in which U6 promoter was inserted. Luciferase reporter assays were performed as described in *SI Materials and Methods*. (*C*) The vectors used in the luciferase reporter assay to show the efficiency of PRDM1 shRNA-mir30 knockdown in 293T cells. (*D*) Demonstration of the efficiency of the PRDM1 shRNA-mir30 construct cotransfected with the luciferase reporter vector. PRDM1 shRNA-mir30 was generated based on manufacturer instructions (Open Biosystems). Empty retroviral vector transfected 293T cells were used for normalization. The result is representative of two independent experiments with two technical replicates. (*E*) Western blotting of vector-only or PRDM1 shRNA-mir30–transduced, GFP-sorted, NKYS cells.

1. Yan J, et al. (2007) BLIMP1 regulates cell growth through repression of p53 transcription. *Proc Natl Acad Sci USA* 104:1841−1846.

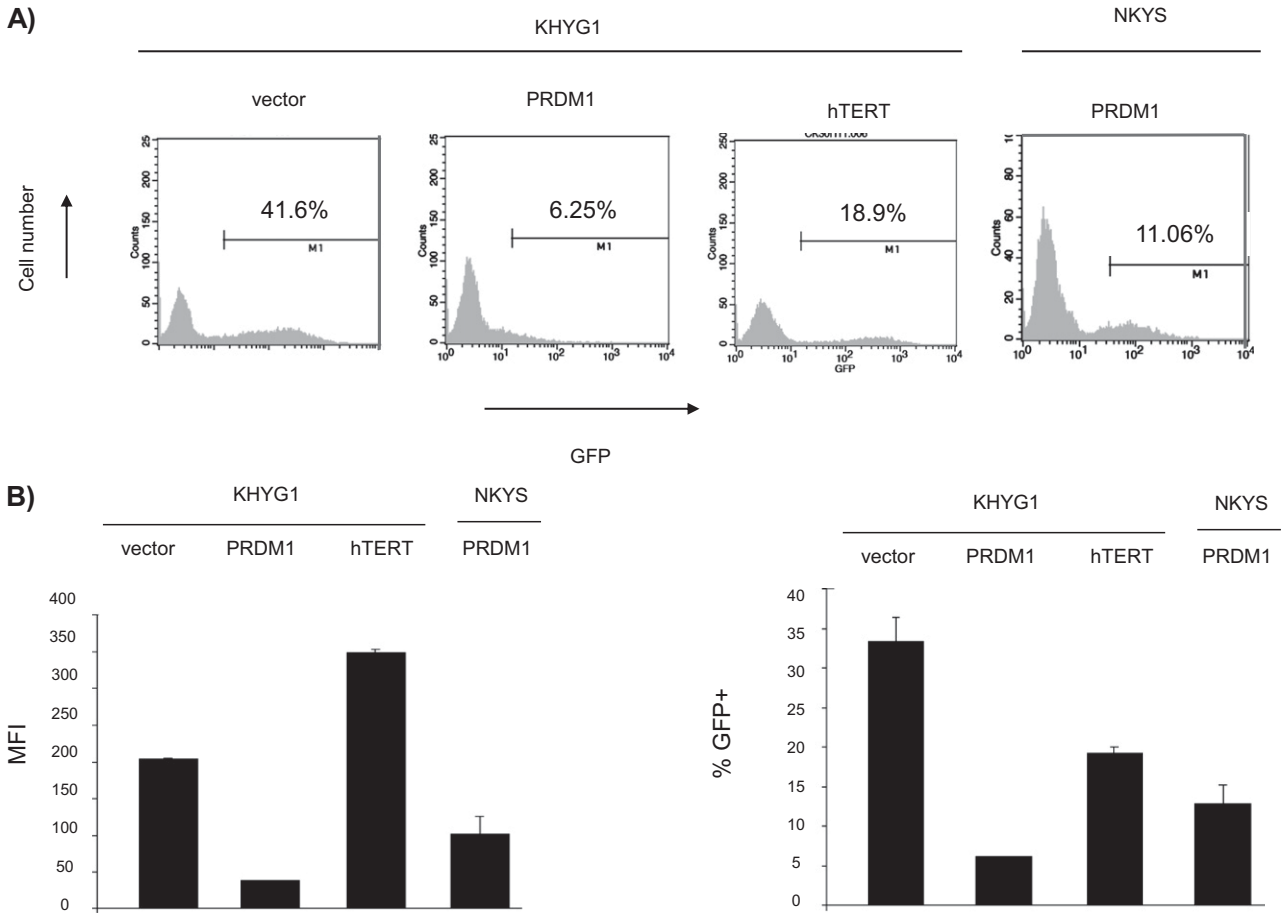

Fig. S8. Comparison of the mean fluorescence intensity and the percentage of GFP in pMIG-, PRDM1-, or hTERT-transduced NK cells. (*A*) FACS profile of vector-, PRDM1-, or hTERT-transduced KHYG1 and NKYS cells 9 d after transduction. (*B*) The mean fluorescence intensity (*Left*) and percentage of GFP(+) (*Right*) in PRDM1- or hTERT-transduced NK cells were compared. The SD bar represents two technical replicates, and vector and PRDM1 transduction in KHYG1 cells are representative of two independent experiments. hTERT and PRDM1 transduction in KHYG1 and NKYS cells, respectively, represents one of two independent experiments.

Table S1. Pathological characteristics of NK cell tumors

Samples	Age	Sex	Biopsy site	CD3e	CD2	CD5	CD7	CD4	CD8	CD56	TiA1	EBV
OM1	48	F	Bone marrow	+		—				+		+
OM2	68	F	Nasal cavity	+		—				+	+	+
OM3	46	F	Nasal cavity	+	+	—	+	—	—	+	+	+
OM4	48	M	Nasal cavity	+						+	+	+
OM5	79	M	Nasal cavity	+	+	—	+	—	—	+	+	+
OM16	66	M	Testis (secondary)	+	+	—	—	—	—	+	+	+
OM7	40	F	Bone marrow	+		—		—	+	+	+	+
OM8	48	F	Small bowel	+	+	—	+	—	—	+	+	—
OM9	57	M	Nasal cavity	+	+	—		—	—	+	+	+
OM10	69	M	Nasal cavity			—		—	—	+		+
OM11	42	M	Testis (secondary)	+	+	—				+	+	+
T12	67	M	Nasopharyngeal	+	+	—	+	—	+	+	+	+
T13	62	M	Nasopharyngeal	—	+	—	—	—	—	+	+	+
T14	72	M	Nasopharyngeal	+	+	—	—	—	—	+	+	+
T15	31	F	Nasopharyngeal	+	+	—	+	—	NA	+	+	+
T16	58	M	Nasopharyngeal	+	+	—	+	—	—	+	+	+
T17	78	M	Nasopharyngeal	+	NA	—	+	NA	+	+	+	+
T18	54	M	Nasopharyngeal	—	+	—	NA	—	—	NA	+	+

NA, not available.

Table S2. Pathological characteristics of NK cell lines

	Patient						
Cell line	Age	Sex	Ethnicity	Original description of disease	CD56	EBV	IL-2 dependency
KHYG1	45	F	Japanese	ANKL	+	—	+
NKYS	19	F	Japanese	ENKL	+	+	+
KAI3	13	M	Japanese	Severe CAEBV	+	+	+
IMC1	42	M	Native American	ANKL	+	—	+
NK92	50	M	Unknown	ANKL	+	+	+